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A Starchless Mutant of *Nicotiana sylvestris* Containing a Modified Plastid Phosphoglucomutase

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ABSTRACT

A mutant (NS 458) of *Nicotiana sylvestris* (Spegazzini and Comes) unable to synthesize leaf starch was isolated in the M_2 generation following ethyl methanesulfonate mutagenesis by testing with iodine. Segregation ratios in reciprocal F_2 progenies showed that the starchless phenotype resulted from a recessive mutation in a single nuclear gene. DEAE-agarose chromatography showed that the mutant is grossly deficient in plastid phosphoglucomutase (EC 2.1.5.1) activity. The structure of the enzyme is changed, as evidenced by increased Michaelis constants and by the prolonged activation period (>40 minutes) observed when the enzyme is assayed in triethanolamine buffer rather than imidazole buffer. The activity of the wild-type enzyme with saturating glucose 6-P alone was 7% of the activity when saturating glucose 1,6-P_2 was also present. The results suggest that glucose 1,6-P_2 is both an effector and a dissociable reaction intermediate. The growth rate of mutant and wild-type plants were not significantly different in continuous light and on an 8-hour dark, 16-hour light cycle and the mutants grew normally under greenhouse conditions. The mutant supports growth during diurnal periods of darkness by vacuolar storage of sugars instead of chloroplast storage of starch. The simplification in metabolism achieved by blocking the diversion of plastid fructose-6-P to starch facilitates the induction of oscillations in CO_2 fixation.

Carbon partitioning in photosynthetic tissues is determined by complex regulatory interactions which are only partially understood (9, 10, 17, 24). The metabolic intermediates being partitioned themselves influence the rates of CO_2 fixation, hence description of the factors limiting net carbon assimilation ultimately requires modeling the system as a whole. In studying complex systems a useful method is to examine a simplified system in which one component has been eliminated or, failing that, modified in a known way. Such an approach requires the isolation of viable mutants with defined lesions in the metabolism of photosynthetic carbon in a species convenient for both biochemical and physiological studies.

A nuclear mutation reducing the activity of plastid PGI[1] by 50% has been recovered in *Clarkia xantiana* (11). The mutant had higher levels of sucrose, lower levels of starch, and reduced leaf weight relative to wild type, indicating that wild-type levels of plastid PGI are required to maintain the normal carbohydrate status and growth rate. Starchless mutants have been obtained in *Arabidopsis thaliana* lacking plastid PGM (1) and ADP-Glc pyrophosphorylase (12). The PGM mutant accumulated high levels of soluble sugars in place of starch and displayed reductions in photosynthesis and growth rate relative to wild type when grown on a 12 h photoperiod. The apparent importance of starch formation in the regulation of carbon metabolism in *Clarkia* and *Arabidopsis* prompted us to search for starchless mutants of tobacco to extend our earlier studies on the partitioning of photosynthetic intermediates (8).

In a mutant blocked in starch synthesis the regulatory interactions of photosynthesis are less complex than in the wild type in that there is a simple inverse relationship between the availability of cytosolic Pi (released in sucrose synthesis) and CO_2 fixation. All the triose-P not exported from the chloroplast in exchange for cytosolic Pi is converted to RuBP so that a low cytosolic Pi leads to high CO_2 fixation, and *vice versa*. In wild-type plants, on the other hand, part of the retained triose-P that is converted to Fru-6-P is partitioned between starch formation and RuBP regeneration. The partitioning is not a stoichiometric ratio but is regulated indirectly by the stromal ratio of 3-phosphoglyceric acid to Pi (17). The more the diversion to starch, the less the coupling between sucrose synthesis and CO_2 fixation.

In this paper, we report the isolation and characteristics of a starchless mutant of *Nicotiana sylvestris* (a diploid species of tobacco) grossly deficient in plastid PGM activity. We show that the starchless phenotype results from a mutation in a single nuclear gene for plastid PGM. The mutant produces an enzyme with elevated Michaelis constants and a long activation period. In contrast to the PGM and ADP-Glc pyrophosphorylase mutants of *A. thaliana* (1, 12), the starchless mutant of *N. sylvestris* grows normally in both continuous light and under natural day-length conditions. We present evidence that soluble sugars, mainly sucrose, are employed as an alternative carbon reservoir in place of starch to support growth during diurnal periods of darkness.

MATERIALS AND METHODS

Mutant Isolation. Seeds for *Nicotiana sylvestris* (Spegazzini and Comes) were soaked in water for 12 h, exposed to 0.4% ethyl methanesulfonate (2 h), and grown to maturity in the field for collection of M_2 seeds as described previously (14). The M_2 seeds ($n \simeq 400$/family) were germinated in closed Petri plates on vitamin-free RN medium (14) containing activated charcoal (Darco G-60, 4 g/L; this appeared to suppress fungal root infections). After 7 d of continuous illumination (800 μE m^{-2} s^{-1}) in normal air the plates were maintained without covers in a similarly illuminated Plexiglas chamber (31 L) flushed (100 mL/min) with an atmosphere of 1% CO_2, 21% O_2. This maximized the accumulation of starch in the cotyledons. On d 9 the plates were flooded with ethanol. This was replaced after about 2 h. Next day, when the Chl had been extracted, the ethanol was replaced by iodine solution (KI, 9 g; concentrated HCl, 22.5 mL; I_2, 0.9 g in 1 L). Normal unshaded cotyledons stained back.

[1] Abbreviations: PGI, phosphoglucoisomerase; PGM, phosphoglucomutase; RuBP, ribulose 1,5-bisphosphate.

Cotyledons that failed to accumulate starch were yellow. Small true leaves were yellow or black. The pale cotyledons of mutants with Chl deficiencies also failed to stain black. Seeds of families that appeared to segregate starchless or low-starch mutants were again germinated and the seedlings transferred to rectangular Petri plates in 6 × 6 arrays. The 7 d old seedlings were grown for 48 h in 1% CO_2, 21% O_2 as before. A cotyledon was removed from each plant and placed in the appropriate well of a serological plate following the array pattern of the Petri plate. The cotyledons were then bleached with ethanol and stained in order to identify mutant plants. Starchless plants of the mutant designated NS 458 were grown in the greenhouse, reciprocally crossed with wild-type plants and also selfed.

Enzyme Assays. The standard assays for PGM (EC 2.7.5.1) and PGI (EC 5.3.1.9) were a modification of Boehringer-Mannheim Co. quality-control procedures. The assay mixtures (2.5 mL) contained triethanolamine HCl (+NaOH) buffer (pH 7.6), 80 mM; $MgCl_2$, 2 mM; EDTA, 0.8 mM; NADP, 0.32 mM; Glc-6-P dehydrogenase (EC 1.1.1.49) from torula yeast (Sigma, 0.88 units/mL, 2.7 μg/mL) and substrate, 2 mM (for the PGM assay, Glc-1-P with 1% Glc-1,6-P_2 [Sigma] and for the PGI assay, Fru-6-P ["less than 1 mol % of Glc-6-P." Sigma]). The reaction was initiated by adding the substrate and followed spectrophotometrically at 340 nm on a Gilford 250 recording spectrophotometer. Better reproducibility was obtained if the dehydrogenase was first freed of $(NH_4)_2SO_4$ by Sephadex G-25 chromatography as ammonium ions inhibit PGM. For both enzymes activity is defined as follows: 1 unit reduces 1 μmol NADP/min at 30° C under the above conditions. For assaying the mutant plastid PGM it was preferable to use imidazole-HCl buffer, pH 7.6, 80 mM in the assay mixture (see "Results").

Separation of Isozymes. Plastid and cytosolic isozymes of PGM from spinach (15) and of PGI from spinach and cauliflower (27) have been separated by chromatography on DEAE-cellulose. In both studies the plastid isozymes were the last to be eluted on increasing the applied KCl concentration. DEAE-cellulose chromatography has also been used to separate isozymes of PGM from yeast (3). We obtained greater separations between plastid and cytosolic isozymes using DEAE-agarose chromatography as follows: Leaf tissue (1.5 g fresh weight) of greenhouse-grown plants was homogenized in 10 mL of K-phosphate buffer (pH 7.4, 10 mM) containing DTE (12 mM). The homogenate was centrifuged at 27,000*g* for 15 min and the supernatant applied to a Sephadex G-25 column (20 × 70 mm, Pharmacia) equilibrated with phosphate buffer (pH 7.4, 10 mM) and DTE (2 mM). The effluent enzyme fraction (11 mL) was applied to a DEAE-agarose column (12 × 230 mm; Bio-Gel A 100–200 mesh, Bio-Rad) previously equilibrated with the same phosphate DTE solution. The column was eluted at 65 mL/h with a linear gradient of KCl (0–500 mM in 300 ml) in the same buffer-DTE solution. The proportion of recovered PGM and PGI activity in the last eluted peaks was enhanced when a chloroplast-enriched preparation of *Nicotinia tabacum* leaves was examined, confirming that these were the plastid isozymes. Plastid PGM for kinetic studies was isolated by this procedure, except that 2 g fresh weight of leaf tissue was used. The effluent peak fractions were freed of KCl by chromatography on a Sephadex G-25 column (20 × 70 mm) equilibrated and eluted with K phosphate buffer (pH 7.6, 10 mM) and DTE (2 mM). Alternatively, they were concentrated by centrifugal filtration and then again concentrated after adding phosphate buffer plus DTE. The enzyme in phosphate buffer plus DTE lost about half its activity on storage overnight at 4° C. It was more stable in phosphate buffer plus DTE than in imidazole buffer plus DTE. A major portion of the protein in these fractions was RuBP carboxylase/oxygenase (see Fig. 1 below). Enzyme with a much higher specific activity (19 units mg^{-1}), but equally unstable, was obtained from *N. tabacum* leaves by a sequence involving $(NH_4)_2SO_4$ precipitation (45–90%), and chromatography on agarose A-15 (Bio-Rad) and on DEAE-agarose. SDS-PAGE showed that this preparation contained a protein with the same mobility as the rabbit PGM marker of 60,600 mol wt (18) and that RuBP carboxylase/oxygenase was absent.

Kinetic Studies of PGM. The reaction contained the same concentrations of buffer (imidazole-HCl or triethanolamine HCl [+NaOH], pH 7.6), $MgCl_2$, EDTA, NADP, and Glc-6-P dehydrogenase used in the standard assay. The dehydrogenase was transferred to 10 mM K phosphate buffer, pH 7.6, by Sephadex G-25 chromatography prior to use. The substrates were K_2 α-D-glucose 1-P ("98–99%, essentially free of α-D-glucose 1,6-P_2," Sigma) and cyclohexylammonium α-D-glucose 1,6-P_2 ("95–100%, synthetic," Sigma). The concentration of the varied substrate, added last, ranged from 0.2 to 30 × K_m^{app}. In determining the K_m for Glc-1-P alone, the range was less as substrate inhibition was observed. Nine data points were collected per determination. In all experiments the PGM was left in the imidazole containing mixture prior to the addition of substrates for about 30 min. in order to allow the imidazole buffer to fully activate the enzyme. For the wild-type enzyme the half-time of activation in 80 mM imidazole buffer was about 5 min. Least-square maximum-likelihood estimates of K_m values and their asymptotic standard errors were obtained by iterative fitting using a BASIC version of an earlier FORTRAN program (7).

Carbohydrate and Organic Acid Contents. Six 4-mo-old greenhouse-grown plants similar in leaf size and general appearance were sampled AM and PM on 5 sunny d during an 11 d period. On these days the maximum temperatures were about 30° C. Three of the plants were wild type and three mutant NS 458 from the original M_2 generation. For a given plant 5 discs, 16 mm diam (total 10 cm^2), were punched from a medium-sized leaf on one side of the midvein at 8:30 AM, frozen in liquid N_2 and stored frozen. At 2 PM the other half of the leaf was similarly sampled. The AM samples from a given plant were combined as were the PM samples. Each combined sample of 25 frozen discs was added to 20 mL of boiling 20% ethanol, homogenized, and centrifuged at 45,000*g* for 7 min. The supernatant was decanted, the residue was suspended in water and again centrifuged. This was repeated to give a combined extract of 50 ml. Dehydrogenase-coupled spectrophotometric assays for citric acid, L-malic acid, glucose, fructose, and sucrose were performed on these extracts using assay kits from Boehringer-Mannheim. The enzymatic procedures proved to be more reliable than chromatographically separating the organic acids and neutral sugars (21) and then assaying these by HPLC. The results for glucose, fructose, and sucrose were expressed as glucose equivalents and combined under the heading "soluble sugars" because a significant amount of sucrose hydrolysis appeared to have taken place in the leaf discs before they were added to the boiling ethanol.

The starch content of the samples was determined by suspending each residue in 0.8 M Na acetate buffer (10 ml), pH 4.9, containing crude α-amylase from *Aspergillus oryzae* (10 mg Mylase ME, U.S. Biochemical Corp.) and shaking the mixture gently for 16 h at 28° C. The suspension was then heated at 100° C for 5 min, centrifuged, and the supernatant decanted. The residue was resuspended in water and again centrifuged; final combined extract, 50 ml. Aliquots (5 ml) were combined from the residue extracts from each plant to yield four samples: wild-type AM and PM, mutant AM and PM. These were applied to columns of SP-Sephadex-C25-H^+ (10 × 50 mm, Pharmacia) and Na-formate equilibrated QAE-Sephadex-A25 (10 × 50 mm, Pharmacia) so coupled that the effluent from the former flowed into the latter (21). The columns were eluted with water. The neutral eluates (50 ml) were concentrated to 400 μL with the aid of a rotary evaporator and filtered prior to HPLC. Aliquots of

these fractions were chromatographed on a Bio-Rad Aminex HPX-87C column, 7.8 × 300 mm, at 80° C, plus anion-OH and cation-H guard columns at 25° C; flow rate 0.6 mL water/min. The chromatographic pump and detector were from LDC (constaMetric III, refractoMonitor) and the column heater was from Bio-Rad. The glucose peak was well separated from earlier minor oligosaccharide peaks and from a small peak with the retention time of xylose.

RESULTS

Mutant Isolation and Genetic Characterization. The procedure for isolating starchless mutants, a modification of that described by Caspar et al. (1), was developed as an adjunct to a search for photorespiratory mutants (14). Advantage was taken of the accumulation of starch that took place in cotyledons when photosynthesizing seedlings (d 7) were transferred to a high CO_2 atmosphere (1% CO_2, 21% O_2) for 2 days. The cotyledons of all normal seedlings on treatment with ethanol to remove Chl and then iodine solution stained black. Starchless leaves were yellow. Of the 683 families of M_2 generation seedlings tested only one had seedlings that showed a clear starchless phenotype in otherwise normal seedlings. Normals and mutants (NS 458) occurred in a 3:1 ratio in the original M_2 family ($\chi^2 = 0.424$, $P = 0.5$), indicating segregation of a single nuclear recessive mutation. Eleven of the plants selected by testing their cotyledons were grown to maturity in the greenhouse, self pollinated, and reciprocally crossed with wild-type plants. The seedlings from all selfed lines were starchless. The F_1 seedlings from the crosses all stained for starch. The starchless character resegregated in the F_2 generation as expected (Table I). Backcrossing of the mutants with wild-type plants as the female parent will be continued through several generations to reduce the chance that observed differences between the mutants and wild-type plant are the result of unrecognized mutations.

Enzymatic Deficiency. Agarose-gel electrophoresis of leaf extracts indicated that the starchless plants lacked the plastid (fastest migrating) isozyme of PGM. Two slower migrating cytosolic isozymes were present. The mutant is thus analogous to the starchless mutant of *A. thaliana* isolated by Caspar *et al.* (1). This conclusion was placed on a quantitative basis by employing DEAE-agarose chromatography. Figure 1 shows a typical elution profile for PGM and PGI from wild-type plants. The plastid isozymes, being the more negatively charged, were the last to be eluted. The multiplicity of the earlier peaks appear to be the result of complex protein-protein or protein-carbohydrate interactions and not an indication of the relative importance of cytosolic isozymes. (When the first PGI peak was relatively high so was the first PGM peak and both coincided with the leading protein peak.) The important observation, from the point of view of the present study, is that the cytosolic PGM activity does not tail into the plastid PGM region but falls to the baseline. When leaves of mutant NS 458 were examined a small but detectable peak was observed in the plastid PGM region. Table II shows the results of replicate determinations on leaves of greenhouse-grown plants. The mean plastid PGM activity for the mutant, based on the rate of reaction in the standard assay at 14 min was

Table I. *Segregation of Starchless Mutants in the F_2 Generation of Mutant NS 458*

Parental cross (F_1)	Observed F_2 Ratio		Pooled χ^{2a}	P[b]
	Normal	Mutant		
NS 458 × wild type	639	194	2.45	>0.3
Wild type × NS 458	634	193		

[a] Tested for 3:1 ratio. [b] P value for *df* 2.

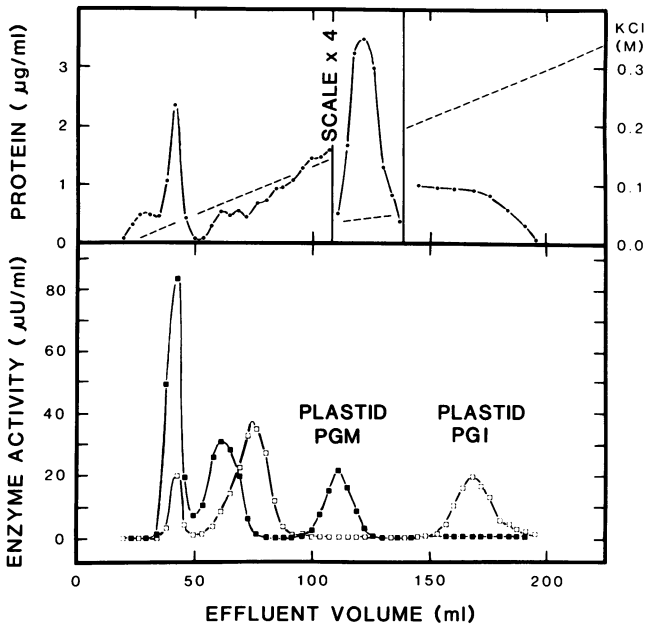

FIG. 1. Separation of cytosolic and plastid isozymes of PGM and PGI from wild-type *N. sylvestris* by DEAE-agarose chromatography. PGM, ■; PGI, □.

1.3% of the mean activity for the wild-type plants (but see next two sections).

The availability of mutant and wild-type plants and heterozygotes from crosses between them makes it possible to inquire whether PGM activity is proportional to the number of functional genes and whether the expression of other genes for starch synthesis and breakdown is influenced by the absence of starch-related intermediates (1, 2). We have confined our attention to the first two enzymes in the pathway of starch synthesis. Table II shows that the differences in PGI activity between wild-type, F_1 heterozygotes, and mutant plants were negligible. Although the plastid PGM results showed more variation, they indicate that the heterozygotes make somewhat more enzyme than would be expected on the basis of one normal gene but less than for two genes. (The wild-type value for two genes is 219 ± 57 milliunits·g fresh weight^{-1}. The probabilities are about 3:1 that the value for the heterozygote, 170 ± 77, differs from this value and about 8:1 that it differs from the one gene value of 109 ± 29.) This apparent departure from proportionality could imply that a decrease in the average level of some later metabolite in starch synthesis brought about by a lower level in plastid PGM leads to a compensatory increase in transcription of the functional gene. However, we cannot rule out the possibility that it is a consequence of some unrecognized mutation in the particular starchless line used for the cross. The problem will have to be reinvestigated with progeny from repeated backcrosses of mutants with wild-type plants.

Changes in the Kinetic Properties of PGM. The kinetic properties of plastid PGM from mutant and wild type were compared to determine whether the mutant makes a small amount of normal enzyme or whether the mutation changed the structure of the enzyme itself. The differences in observed properties establishes that the mutation alters the structure of plastid PGM.

The most striking difference is the activation lag observed in the standard assay using 80 mM triethanolamine buffer, pH 7.6. For the wild-type enzyme, when the reaction was initiated by adding Glc-1-P containing Glc-1,6-P_2, there was a variable short delay of about 1 min before a steady-state rate of NADP reduction was observed. For the mutant enzyme the activation period extended to at least 40 min; at 14 min the rate was about 60%

Table II. *Activities of Cytosolic and Plastid Isozymes of Phosphoglucomutase and Phosphoglucoisomerase in Leaves of Greenhouse-Grown Wild-Type and Mutant NS 458 Plants as Determined by DEAE-Agarose Chromatography (Fig. 1)*

Means ± SD listed are uncorrected for losses of activity on chromatography.

Strain	Enzyme Activities[a]			
	Phosphoglucoisomerase		Phosphoglucomutase	
	Cytosol	Plastid	Cytosol	Plastid
	milliunits·g fresh wt^{-1}			
Wild type (n = 5)	292 ± 106	228 ± 20	643 ± 83	219 ± 58
NS 458 × Wild type (n = 3)	405 ± 164	256 ± 40	752 ± 160	183 ± 92
Wild type × NS 458 (n = 3)	368 ± 102	234 ± 18	535 ± 85	158 ± 76
NS 458 selfed (n = 3)	401 ± 34	291 ± 22	589 ± 31	2.9 ± 0.1

[a] One unit of activity reduces 1 μmol NADP min^{-1} at 30° C in the standard assay (see "Materials and Methods"). Mean recoveries of applied activity (in row order) were for PGI: 60, 75, 77, 70% and for PGM: 51, 69, 59, 58%. Mean conversion factors (n = 14): fresh wt/area, 20.7 ± 1.9 mg cm^{-1}; dry wt/area, 3.20 ± 0.37 mg cm^{-1}; fresh wt/dry wt, 6.5 ± 1.3.

of the rate at 40 min. The lag was unchanged if there was a delay of 40 min between adding Glc-1,6-P_2 and Glc-1-P to the reaction mixture, but it decreased to about 6 min when the procedure was reversed. The lag was eliminated for both mutant and wild-type enzymes when the reaction mixture contained imidazole buffer instead of triethanolamine buffer.

The above activation phenomenon is probably related to the activation observed with imidazole buffer. The steady-state of NADP reduction observed in imidazole buffer was a function of the time that PGM was in contact with the buffer prior to addition of the substrate. For 80 mM buffer the half-time for wild-type enzyme was about 5 min, the overall activation being greater than twofold. The half-time decreased as the buffer concentration increased. This phenomenon has been observed with PGM from rabbit muscle and other sources (19, p. 439). Imidazole is considered to promote the dissociation of inhibiting divalent metal ions by coordinating to the enzyme-bound ions. These results therefore, provide indirect evidence that the plastid enzyme is structurally related to the rabbit enzyme and that they share a common mechanism (indirect phosphate transfer via Ser[116] [13, 18–20]). The *Micrococcus lysodeikticus* and *Bacillus cereus* enzymes, which are believed to function by direct phosphate transfer, do not show imidazole activation (6, 19).

Mutant and wild-type PGM showed normal Michaelis-Menten kinetics for the 'forward' direction (Glc-1-P → Glc-6-P) without evidence for significant inhibition at high substrate concentrations. Table III shows that their K_m values, determined in imidazole buffer under identical conditions, are very different. For the wild-type enzyme the concentrations of the constant substrates were saturating, hence the true K_m values were equal to their apparent values. For the mutant they were a little less than saturating. True K_m values were calculated by assuming that the plastid enzyme, like the rabbit (20), yeast (3), and *Escherichia coli* (6) enzymes obeys Ping-Pong kinetics (22, p. 606). The correction is not large and it would probably be similar even if the kinetics were of the Ordered Bi Bi type encountered with the *M. lysodeikticus* and *B. cereus* enzymes (6; 22, p. 560).

Relationship of Kinetic Constants to Starch Synthesis. The fractions assayed from the DEAE-agarose column contained KCl and the standard assay used triethanolamine buffer instead of imidazole buffer. As the final concentrations were about 50 mM KCl and 80 mM triethanolamine, K_m^{app} values were determined for this condition. The K_m^{app} values for the mutant PGM were based on the reaction velocity after a standard 14 min activation time. K_m values were calculated for the mutant PGM assuming Ping-Pong kinetics as in Table III. For Glc-1-P the K_m values, wild type then mutant, were 65 μM and 650 μM and for Glc-1,6-P_2 they were 2 μM and 10 μM. Omitting the extra KCl lowered the K_m values by 50% for Glc-1-P and by 90% for Glc-1,6-P_2. (This difference in response to changing the ionic strength is consistent with the view that charge interactions play an important role in sequestering Glc-1-6,P_2 within the cleft of the dephosphorylated enzyme [13].) On the basis of the above K_m determinations, the combined factor for the mutant to correct for lack of substrate saturation in the standard assay and for incomplete activation at 14 min is about 3. The corrected activity for Table II is thus 8.7 milliunits·g fresh weight^{-1}; 4% of the wild-type activity.

Table III. *Michaelis Constants for Plastid PGM from Wild-Type and Mutant NS 458 Plants Determined in 80 mM Imidazole Buffer, pH 7.6*

The asymptotic standard errors of the K_m determinations were about ± 10% of the estimated values listed.

Strain	Constant	For Glc-1-P		For Glc-1,6-P_2	
		K_m^{app} or K_m	(Concentration Glc-1,6-P_2)	K_m^{app} or K_m	(Concentration Glc-1-P)
		μM	μM	μM	μM
Wild type	$K_m = K_m^{app}$	30	(100)	0.53	(1,200)
NS 458	K_m^{app}	400	(100)[a]	18	(12,000)[a]
	K_m	475		19	

[a] The concentration of the constant substrate was not saturating. True K_m values were calculated by assuming Ping-Pong kinetics (19; 22, p. 606): $K_{m(A)} = K_{m(A)}^{app}\,(1 + K_{m(B)}/[B])$ and $K_{m(B)} = K_{m(B)}^{app}\,(1 + K_{m(A)}/[A])$.

The above kinetic constants are for the 'forward' direction Glc-1-P → Glc-6-P whereas starch synthesis proceeds in the reverse direction. If the mutation influences only the structure of the enzyme so that the same amount of enzyme protein is present in mutant and wild-type plants, the V_{max} values for Glc-1-P should be in the ratio of the observed activities, *i.e.* $V_{max(Glc\text{-}1\text{-}P)}$ for the mutant is about 4% of the value for the wild-type enzyme. The Haldane relationship, assuming Ping-Pong kinetics, requires that a decrease in $V_{max}^2/K_{m(A)}\cdot K_{m(B)}$ for one direction is accompanied by a proportional decrease in the analogous expression for the other (22, p. 616). It is probable, therefore, that for the direction of starch synthesis V_{max} is substantially reduced and both $K_{m(Glc\text{-}6\text{-}P)}$ and $K_{m(Glc\text{-}1,6\text{-}P_2)}$ are increased. Such changes suffice to explain why the mutant does not accumulate starch. A small amount of starch could be formed, but it would usually be removed by degradative enzymes.

Glc-1-P as a Single Substrate. In the forward reaction for PGM, as formulated for the rabbit muscle enzyme (19, 20), Glc-1-P reacts with the phospho-enzyme, E-OP, to yield an intermediate complex involving three distinct states: [E-OP + Glc-1-P $\leftrightharpoons$ E-OH + Glc-1,6-P_2 $\leftrightharpoons$ E-OP + Glc-6-P]. The reaction is completed by release of Glc-6-P. The Glc-1,6-P_2 may, but need not, dissociate from the active site before again reacting with the enzyme. The concentration of Glc-1,6-P_2 in the reaction mixture thus determines the amount of E-OP available. The rabbit enzyme as isolated is in the phosphorylated state, hence on adding Glc-1-P alone Glc-1,6-P_2 is formed at the active site. Part of this dissociates and part yields Glc-1-P which can then dissociate. The overall conversion of Glc-1-P to Glc-6-P proceeds at a rate determined by the very low steady-state concentration of Glc-1,6-P_2. For a saturating Glc-1-P concentration the ratio (rate without Glc-1,6-P_2)/(rate with saturating Glc-1,6-P_2) should fall to zero as the number of enzyme units present (the denominator) decreases. A large decrease was observed and the shape of the experimental and predicted curves agreed, but the experimental curve approached a limiting ratio of about 0.7% rather than zero (20). In the limit, therefore, the enzyme behaved as if k_{cat} was reduced to 0.7% of its normal value and the K_m for Glc-1,6-P_2 was zero, *i.e.* no Glc-1,6-P_2 escaped from the active-site cleft during the reaction. The possibility was considered that a small amount of a second enzyme that did not require Glc-1,6-P_2 was present. Our experiments indicate that the plastid PGM has similar properties.

In the course of determining the K_m values listed in Table III it was noted that Glc-1-P alone sufficed to produce a slow rate of reaction with the wild-type enzyme. Such a phenomenon has been reported for PGM from pea and mung beans (4). Plastid PGM, concentrated by centrifugal filtration was assayed in imidazole buffer after a 30 min activation period, first with 1.2 mM Glc-1-P and then with added 0.1 mM Glc-1,6-P_2. The sensitivity of the spectrophotometer allowed 6 enzyme concentrations to be used differing by a factor of two (32-fold range). The ratios of the two activities showed appreciable scatter, however there was no discernible decrease in the ratio as the enzyme concentration increased and no significant difference between enzyme derived from DEAE-agarose chromatography with and without a prior $(NH_4)_2$ SO_4 fractionation step. The mean Glc-1-P activity was 6.8 (±2.0)% of the activity with both substrates (n = 26, data pooled from three experiments). This appeared to be a limiting activity analogous to that observed for the rabbit enzyme, but larger. Activity was also observed with Glc-1-P purified by additional ion-exchange chromatography in this laboratory, hence the result cannot be attributed to Glc-1,6-P_2 present in the commercial sample of Glc-1-P. It is possible that a discernible increase in activity towards Glc-1-P with increasing enzyme concentration was not observed because the highest enzyme concentration tested was not large enough to produce a significant [Glc-1,6-P_2]/K_m^{app} ratio (20). The K_m for Glc-1-P alone was 3.0 (±0.4) μm. The same result was obtained with prior activations in imidazole buffer of 10 and 40 min. The results cannot be explained by the presence of a second enzyme that does not require Glc-1,6-P_2, because in the mutant plants the activity of the second enzyme would be as important as the mutant enzyme with its diminished activity. They may imply that Glc-1,6-P_2 acts both as a dissociable intermediate and as an effector. The intermediate cannot dissociate unless Glc-1,6-P_2 is bound to an effector site.

Growth Comparison of Wild-Type and Mutant Plants. Eighteen mutant plants isolated from the original M_2 family were compared with nine wild-type plants. The mutants showed no obvious abnormalities in the growth room, hence it is probable that any other mutations segregating in this population have little influence on growth under the conditions of the experiment. The plants from d 22 (fifth true leaf emerging for mutants and wild type) were maintained in continuous light equivalent to a dull day in winter (350 μE m^{-1} s^{-1}) with a temperature cycle of 8 h 9° C, 16 h 19° C. On d 27 and 3 days thereafter the lengths and widths of their seventh true leaves were measured. The leaves were then removed and their areas and dry weights determined. The regime was then changed so that the 8 h period at the lower temperature was also dark, and leaf sampling was repeated for the eighth or ninth true leaf.

Table IV shows the results of these experiments. The ratios of area to (length × width) showed no trends with area and no detectable difference between wild-type and mutant for the continuous-light experiments. The (length × width) measurements were therefore converted to area values using the mean factors listed. A linear relationship between area$^{1/2}$ and days described leaf expansion over the four day period studied, *i.e.* the rate of growth in a given direction was constant. If a = area, d = days, the empirical relationship can be expressed as:

$$a^{1/2} = C(d + D) \quad (1)$$

implying

$$a = C^2(d + D)^2 \quad (2)$$

Whereas C, the coefficient of linear expansion, is an empirical constant (cm/day) for a given set of plants, D varies from plant to plant. Table IV shows that the coefficients of area expansion, C^2, for dark-cycle plants were 9 to 12% of those for plants grown in continuous light. The difference between wild-type and mutant plants was significant only for the 8 h dark cycle. The ratios of dry weight to area also showed no trends with area in the two parts of the experiment. These ratios were greater for the wild-type plants. The 2.5-fold greater ratio for the dark-cycled plants offset the difference in area expansion. The coefficients of dry weight increase for the dark-cycled plants were about 28% of those for plants grown in continuous light. There was no significant difference in dry weight increase per day between wild-type and mutant plants.

Measurements of leaf growth integrate net photosynthetic assimilation, dark respiration losses, and transport from the leaf. The above experiment indicates that for the conditions studied metabolism is so regulated that these processes are independent of the mode of storage of assimilated carbon. This conclusion is probably also valid for shorter daylength conditions. No difficulty was encountered in growing the starchless plants in the greenhouse. Even during the winter months when the day length was between 10 and 12 h, M_2-mutant and wild-type plants were indistinguishable and reached flowering at the same time.

Carbohydrates and Organic Acids. The above results imply that the diurnal-storage function of starch has been taken over by other metabolites. Table V shows a comparison of sets of three wild-type and three mutant plants sampled on five sunny days. Leaf discs were punched from the same leaves at 9 AM and 2 PM. Samples for different days were combined and, for the starch assay, samples derived from different plants. The wild-type plants showed a 3.7-fold increase in starch content during the day, whereas the starch content of the mutant was negligible. The mutant stored significantly more malic acid than the wild-type plants, but there was no significant difference between morning and afternoon values. Citric acid was similar in wild-type and mutant plants and decreased in the afternoon. It appears, therefore, that the stored soluble sugars, mainly sucrose, account for the ability of the mutant plants to grow normally. The PM–AM difference in glucose equivalents of starch and soluble sugars for the mutant were 84% of that for the wild-type plants. A resultant difference in dry weight of this magnitude might not be obvious for greenhouse-grown plants. In an earlier experiment, in which the greenhouse temperatures were higher

Table IV. *Growth Comparisons of Wild-Type and Mutant NS 458 in Continuous Light and 16-h Light, 8-h Dark Regimes*

Results are means and SD for wild-type (n = 8) or mutant (n = 16) plants, or for all plants if the separate means were almost identical and pooling seemed appropriate.

Regime[a] and Strain	$\frac{\text{Area}}{L \times W}$	$\frac{\Delta \text{ Area}^{1/2}}{\Delta \text{ Days}}$ C	Coefficient of Area Expansion C^2	$\frac{\text{Dry wt}}{\text{Area}}$	Coefficient of Dry Wt Increase
		cm d^{-1}	*cm d*$^{-1}$	*mg cm*$^{-2}$	*mg d*$^{-1}$
Continuous light					
Wild type				1.71 ± 0.15	1.8 ± 0.2
(pooled data)	0.647 ± 0.041	1.01 ± 0.10	1.04 ± 0.21		
NS 458				1.61 ± 0.77	1.7 ± 0.3
16-h light, 8-h dark					
Wild type	0.683 ± 0.022	0.33 ± 0.08	0.11 ± 0.06	4.28 ± 0.31	0.48 ± 0.25
NS 458	0.646 ± 0.041	0.40 ± 0.09	0.13 ± 0.07	3.54 ± 0.41	0.47 ± 0.26

[a] The growth room temperature cycle was 16 h at 19°C and 8 h at 9°C. The dark period coincided with the lower temperature.

Table V. *Carbohydrate and Organic Acid Content of Leaves of Greenhouse-Grown Wild-Type and Mutant NS 458 Plants*

Means ± SD values listed are for sets of three plants. Samples were collected on 5 d and pooled for each plant. Samples from all three plants were pooled in determining glucose from starch.

Strain and hour of sampling	Starch[a] (glucose equivalent)	Soluble Sugars[b] (glucose equivalent)	Malic Acid	Citric Acid
		nmol cm^{-2}		
Wild type				
8:30 AM	275	249 ± 37	396 ± 199	189 ± 41
2:00 PM	1030	483 ± 50	440 ± 48	133 ± 45
NS 458				
8:30 AM	14	263 ± 63	651 ± 174	172 ± 41
2:00 PM	22	1086 ± 121	658 ± 145	109 ± 27

[a] The residues of the leaf extracts on enzymatic treatment also released small, but relatively constant, amounts of xylose: in column order 95, 85, 100, and 150 nmol cm^{-2}. [b] Soluble sugars = Glc (free + in sucrose) + Fru (free + in sucrose). The mean Glc/Fru ratios were AM, 1.19; PM, 1.44. PM > AM > 1, $P < 0.005$.

and samples from different plants were pooled, similar trends to those recorded in Table V were observed despite the fact that the plants were under stress during the hottest part of the day.

The distribution of carbon reserves in leaves of mutant plants grown in continuous light has not been investigated, however, some starch appears to accumulate in leaves of 5 week old plants grown under these conditions. A small but detectable layer of starch was observed in the pellet when the chloroplast fraction obtained from such leaves was disrupted and centrifuged.

The soluble sugars in the experiments recorded in Table V were determined enzymically. The above calculation thus ignores oligosaccharides which may contribute to the ability of the mutant to compensate for the lack of starch storage. Semiquantitative evidence was obtained by HPLC examinations of neutral-sugars fractions from the killed leaf-disc extracts. Several oligosaccharide peaks, possibly fructans, overlapped with the sucrose peak. The refractometer trace indicated that the peaks were greater in the PM samples and that the AM–PM difference was greater for the mutant than the wild-type plants. Their contribution to the alternative storage appeared to be less than that of sucrose. Fructans, like sucrose, are stored in the vacuole (16), hence the overall phenomenon is a replacement of chloroplast storage by vacuole storage.

DISCUSSION

Segregation patterns in reciprocal F_2 progenies (Table I) show that the starchless phenotype of NS 458 results from a recessive mutation in a single nuclear gene. The DEAE-agarose chromatography experiments show that mutant NS 458 is grossly deficient in plastid PGM activity (Table II). When assayed in the forward direction (Glc-1-P → Glc-6-P) the mutant enzyme has higher K_m values for both Glc-1-P and Glc-1,6-P_2 than the wild-type enzyme (Table III), suggesting that the mutation is located in the structural gene for plastid PGM. Antibodies are not yet available to measure the amount of enzyme protein formed in the mutant, however, if gene expression is not influenced by the mutation, V_{max} is about 4% of the wild-type value. It can be inferred that the K_m values are similarly increased and V_{max} decreased for the direction of starch synthesis. The inability of the mutant to accumulate starch can be fully explained in terms of changes in the kinetic constants of PGM. In the mutant PGM limits the flux towards synthesis whereas in wild-type plants the next step, ADP-Glc pyrophosphorylase, is limiting (17).

Rabbit muscle PGM has been extensively studied in terms of mechanism (19, 20), amino-acid sequence (18), and 3-dimensional structure (13). It is therefore of interest to know whether the plastid-PGM has the same mechanism as the rabbit enzyme or whether it resembles PGM from *B. cereus* and *M. lysodakticus* (6). (The mechanism of the rabbit-type PGM involves a phosphorylated enzyme, whereas the *B. cereus* type appears to involve direct phosphate transfer.) The observations concerning the effect of imidazole on plastid PGM activation, the influence of ionic strength on the K_m for Glc-1-P_2, the apparent ability of this enzyme to convert Glc-1-P to Glc-6-P without added Glc-1,6-P_2, and the available mol wt data (see "Materials and Methods") (15, 18), suggest that the plastid enzyme is related to the rabbit enzyme. If this is correct, it still remains to fully explain the role of Glc-1,6-P_2 as a dissociable intermediate both *in vitro* (as discussed above) and *in vivo* (4).

Starch, sucrose, fructans, and organic acids are used by plants to support growth during diurnal periods of darkness. Whereas starch is stored in chloroplasts, sucrose, fructans, and organic acids accumulate in the vacuole. Leaves of *N. tabacum* usually have a high starch content, a negligible fructan content, and a high starch to sucrose ratio (16, 25, 26). The leaves accumulate large amounts of malic acid and less critic acid (25, 26). *N. sylvestris* has similar characteristics. In contrast, barley and other temperate grasses accumulate little starch but store sucrose and fructans (16). Eliminating starch synthesis from *N. sylvestris* thus forces the plants to rely on storage in the vacuole. It appears that

for greenhouse-grown *N. sylvestris* plants (Table V) enhancement in the mutant of diurnal sucrose storage, together with some oligosaccharide storage, approaches equivalence to starch storage in the wild type. The lack of diurnal variation in malic acid content contrasts with spinach where there are large changes in the vacuole storage of both sucrose and malic acid (5). *N. sylvestris* is much more able to compensate for lack of starch storage than *A. thaliana* (1). In both cases the PGM-deficient mutant and wild-type plants grew in continuous light at the same rate (Table IV). Whereas the *N. sylvestris* mutant grew at much the same rate as wild-type plants when the day-length in the greenhouse was 10 to 12 h, the growth rate of the *A. thaliana* mutant was less than one-third that of wild-type plants when the plants were grown on a 12-h dark, 12-h light cycle.

The primary goal of the study was to obtain plants in which photosynthetic metabolism is simplified because the partitioning of Fru-6-P between starch formation and RuBP regeneration is eliminated. The measurements of leaf growth in continuous light (Table IV) suggest that regulatory interactions make starch synthesis and triose-P export equivalent as far as net CO_2 assimilation is concerned. Direct measurements of net CO_2 assimilation rates in normal air (31° C and 500 μE m^{-2} sec^{-1}) for leaf discs of greenhouse-grown mutant plants gave values that averaged 90% of those for wild-type plants. A dramatic difference between leaf discs of wild-type and mutant plants was observed, however, when the metabolism was further simplified by reducing photorespiration to a low level, *e.g.* 1% O_2, 340 μL CO_2/L (32° C, 600 μE m^{-2} s^{-1}). After a 4 min dark period the CO_2 assimilation rate for wild-type leaf discs showed a minor peak followed by a smooth return to a steady-state value (less than 10 min). In contrast, the mutant showed a sharp peak exceeding the initial steady-state value followed by a 4 min trough at about 40% of the steady-state value and then a series of damped oscillations with a period of about 6 min. The oscillations were still detectable after 50 min.

Oscillations in photosynthetic metabolism have been the subject of extensive investigation (23, 24). They are more readily induced in species which accumulate little or no starch, such as barley (23). The starchless *A. thaliana* mutant is reported to have a lower CO_2 threshold for oscillatory behavior than the wild type (23). As noted in the Introduction, eliminating the diversion of stromal Fru-6-P to starch synthesis establishes a direct connection between the triose-P that is not exported from the chloroplast and RuBP regeneration. Under zero-photorespiration conditions the rate of regeneration is equal to the rate of CO_2 fixation. As the triose-P exported is exchanged for Pi, the availability of Pi in the cytosol inversely influences the rate of CO_2 fixation. Studies of oscillations of O_2 evolution and Fru-2,6-P_2 levels in barley suggest that the delay time in the regulation of cytoplasmic sucrose synthesis by Fru-2,6-P_2 accounts for oscillations in the conversion of triose-P to Pi and hence for oscillations in photosynthesis (24). Such an explanation requires that the pulses of triose-P production in the chloroplast have the same frequency as pulses of Pi release in the cytosol, though the phase may be shifted. The importance of the starchless *N. sylvestris* mutant described here is that it provides an experimental subject designed for studying this complex phenomenon. In particular, the prolonged oscillatory period in *N. sylvestris* represents a distinct advantage for photosynthetic studies, and starch accumulation is not merely low, as in barley, but zero.

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